Interaction of Calcium and Vanadate with Fluorescein Isothiocyanate Labeled Ca^{2+}-ATPase from Sarcoplasmic Reticulum: Kinetics and Equilibria

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ABSTRACT: The interaction of Ca^{2+} and vanadate with fluorescein isothiocyanate (FITC) labeled sarcoplasmic reticulum (SR) Ca^{2+}-ATPase has been studied by following the kinetics of changes in the reporter group fluorescence and equilibrium fluorescence levels. The vanadate species bound to the enzyme is clearly monomeric orthovanadate, probably $H_2VO_4^-$. Vanadate binding is noncooperative, suggesting an absence of interactions between the Ca^{2+}-ATPase subunits. The fluorescence experiments confirm the existence of a calcium–enzyme–vanadate complex (in the presence of magnesium). On the basis of the fluorescence properties of this complex, it is similar in its conformation to the calcium–enzyme complex, i.e., "E_1-like" rather than "E_2-like". However, Ca^{2+} binds to the enzyme–vanadate complex via sites that are only accessible from the interior of the SR vesicles. The complex Ca_2E^*Van, which is rapidly formed, isomerizes very slowly ($t_{1/2} \sim 1$ min) to the stable ternary complex. The mutual destabilization between bound vanadate and two bound Ca^{2+} ions is only 1.6 kcal/mol, much smaller than that produced by the interaction of calcium and phosphate.

Vanadate ion (mononuclear hydrated pentavalent V) has been found to be a potent inhibitor of a number of ATPases, as well as other phosphatases (Van Etten et al., 1974; Lopez et al., 1976; Cantley et al., 1978; O'Neal et al., 1979), and is thought to form with them complexes analogous to the phosphoenzymes or to transition states on the pathway for phosphoenzyme hydrolysis (Cantley et al., 1978; Kustin & Macara, 1982; Boyd & Kustin, 1984). The sarcoplasmic reticulum (SR) Ca^{2+}-ATPase is one of the enzymes which binds vanadate strongly (K_d near 1 μM under appropriate conditions) (O'Neal et al., 1979; Dupont & Bennet, 1982; Pick, 1982; Pick & Karlish, 1982; Dux & Martonosi, 1983; Medda & Hasselbach, 1983; Inesi et al., 1984; Ortiz et al., 1984; Highsmith et al., 1985; Andersen & Moller, 1985; Markus et al., 1986). Although a number of researchers, using a variety of methods, have reported that vanadate binds exclusively to the calcium-free form of the Ca^{2+}-ATPase (O'Neal et al., 1979; Pick, 1982; Dux & Martonosi, 1983; Medda & Hasselbach, 1983; Andersen & Moller, 1985), there is good evidence that calcium and vanadate binding are noncompetitive (Inesi et al., 1980; Highsmith et al., 1985; Markus et al., 1986). We have found, by using the initial velocity of the ATPase reaction as a measure of the binding equilibria (Markus et al., 1986), that, in addition to the calcium-free complex EVan[1] with dissociation constant $K_v = 0.4$ μM, there is also a Ca^{2+}–enzyme–vanadate complex, characterized by an equilibrium dissociation constant which is more than an order of magnitude higher ($K'_v = 7$ μM). We report here quantitative studies of the fluorescence intensity changes upon calcium or vanadate addition to SR vesicles labeled with FITC, which have confirmed our conclusions based on the initial velocity of the ATPase reaction (Markus et al., 1986). FITC-labeled SR vesicles respond to calcium addition with a fast quenching of fluorescence intensity, while the addition of vanadate leads to a slow increase in fluorescence intensity (Pick, 1982; Pick & Karlish, 1982; Highsmith et al., 1985). While our previous experiments using the ATPase activity unequivocally demonstrated that Ca^{2+} and vanadate bind noncompetitively to the ATPase itself, the present study provides further information concerning conformational changes (changes in the fluorophore environment) in the species arising from interactions between SR vesicles, calcium, and vanadate, as well as on the nature of the calcium sites involved. A preliminary report of some of these results has been published (Markus et al., 1988).

Materials and Methods

Materials

Fluorescein isothiocyanate (FITC), EGTA, and MOPS were obtained from Sigma, St. Louis, MO; $CaCl_2$, KCl, and $MgCl_2$ (all of analytical grade) were from Merck, Darmstadt; sodium orthovanadate ($Na_3VO_4 \cdot 14H_2O$) was from BDH, Poole, U.K. Ionomycin was obtained from Calbiochem-Hoechst (Lucerne).

Sodium orthovanadate stocks were prepared at a concentration of 1 mM in distilled water, at least 2 days prior to use, to ensure that the equilibrium for dissociation of vanadate to monomer was reached (Kustin & Macara, 1982).

Methods

Sarcoplasmic reticulum vesicles were prepared from rabbit back and hind leg white muscle by a modification (Pickart & Jencks, 1982) of the procedure of MacLennan (1970), suspended to a final concentration of 20 mg/mL in a buffer containing 0.4 M sucrose and 5 mM Tris–SO_4 at pH 8.0, and stored at −70 °C.

Labeling of SR vesicles with FITC was carried out by the method of Pick and Karlish (1982). FITC (dissolved in dimethylformamide) was added to the vesicles suspended in a

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[1] Abbreviations: Van, orthovanadate; SR, sarcoplasmic reticulum; FITC, fluorescein isothiocyanate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; E, Ca^{2+}-ATPase.

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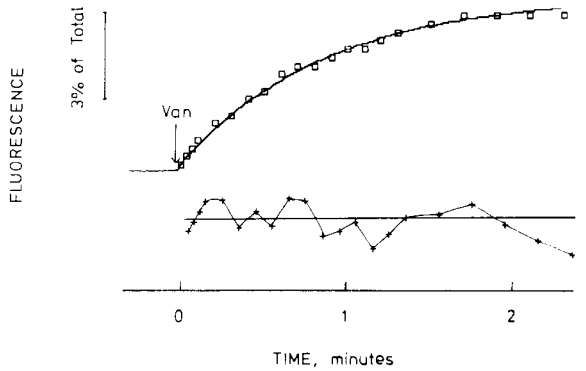


FIGURE 1: Fluorescence intensity increase upon addition of vanadate to FITC-labeled vesicles. Vesicles were incubated for 10 min at 25 °C in 2.5 mL of a mixture containing 100 mM KCl, 5 mM $MgCl_2$, 0.3 mM EGTA, and 200 mM MOPS, pH 7.0. The fluorescence emission at 516 nm was recorded (λ_{ex} = 496 nm). At the time indicated by the arrow, 15 μL of 0.5 mM sodium orthovanadate was added (final concentration 3 μM). The line is the best fit to $F = F_0 + (F_\infty - F_0)(1 - e^{-kt})$. F is the observed fluorescence intensity at time t, and k = 1.16 min^{-1}. The lower trace is the residuals on an enlarged scale.

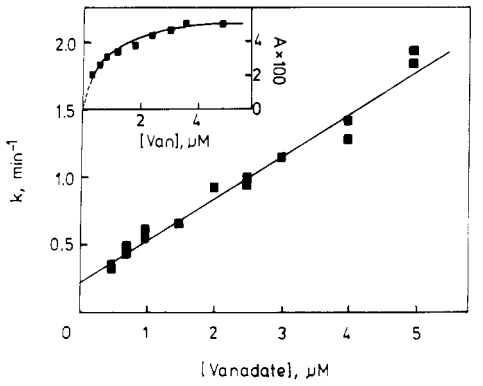


FIGURE 2: Dependence on vanadate of observed rate constants for the fluorescence increase. The pseudo-first-order rate constants determined from the time course of the increase in fluorescence intensity (see Figure 1) at pH 7.0 are plotted as a function of vanadate concentration. The straight line is the best fit to $k = k_{-1} + k_{+1}$[vanadate], with $k_{+1} = (3.1 \pm 0.1) \times 10^5$ M^{-1} min^{-1} and $k_{-1} = 0.21 \pm 0.03$ min^{-1}. (Inset) The *extrapolated* amplitudes [$A = (F_\infty - F_0)/F_0$] of the exponential fluorescence increase (Figure 1) as a function of [vanadate]. The line is the best fit to a binding isotherm, K_v = 0.62 ± 0.01 μM.

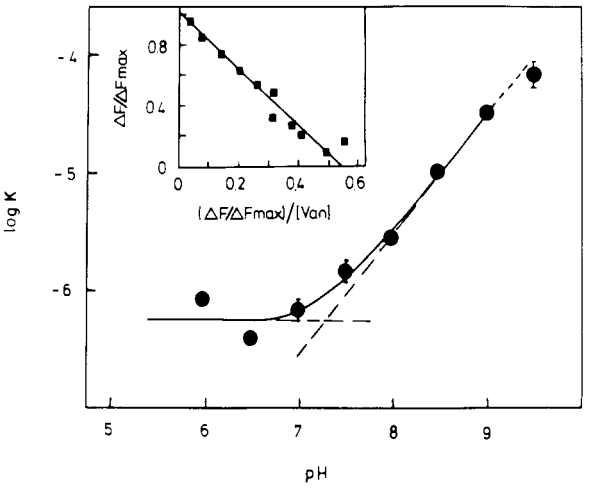


FIGURE 3: pH dependence of the apparent dissociation constant for vanadate (K_v). At each pH, the apparent dissociation constant was determined by addition of various concentrations of vanadate to FITC-labeled SR vesicles and by measurement of the fluorescence change at equilibrium (after more than 10 min). (Inset) Example of data at pH 7.5, in the form of a Scatchard plot.

25 mM Tris–25 mM glycine buffer at pH 9.0, in the presence of 0.1 mM EGTA and 0.2 M sucrose. After 10 min, the vesicles were separated from unbound FITC on a Sephadex G-25 (medium) column according to Penefsky (1977). Under these labeling conditions, the SR vesicles become permeable to Ca^{2+} (Duggan & Martonosi, 1970; Berman, 1982). All experiments reported here use such vesicles, unless otherwise indicated.

Relatively impermeable labeled vesicles were prepared by carrying out the reaction with FITC at pH 7.0, in the presence of 0.2 M sucrose and 50 mM Tris, for 30 min.

Protein assays were performed according to the method of Lowry et al. (1951) with bovine serum albumin (Sigma) as reference. No interference with this assay by FITC was noted.

Free Ca^{2+} concentrations were calculated from association constants of Schwarzenbach et al. (1957). Differences in absolute calcium affinities between this and other published results may be due to the use of different values for the Ca^{2+}–EGTA association constant. The endogenous calcium content in the reaction mixture, derived from SR vesicles and reagents, was assumed to be about 15 μM.

Fluorescence measurements were carried out on a Perkin-Elmer MPF-2A spectrofluorometer with a thermostated cell compartment held at 25.0 ± 0.5 °C. Excitation and emission wavelengths of 496 and 516 nm, respectively, were employed. Fluorescence intensities of suspensions of FITC-labeled SR vesicles were measured with 4–10 μg of protein/mL, in reaction mixtures containing 100 mM KCl, 5 mM $MgCl_2$, 0.3 mM EGTA, and 200 mM MOPS or Tris–maleate buffer. Initial fluorescence levels were measured after 10 min of temperature equilibration in the cell, and the changes in intensity upon addition of $CaCl_2$ or Na_3VO_4 were recorded. In equilibrium experiments, the final observed changes were normalized to the initial fluorescence values (prior to the last reagent addition).

Data Analysis. The parameters for the fit of experimental data to theoretical equations were determined with a grid-search nonlinear least-squares program (Bevington, 1969) written in Applesoft Basic for an Apple II+ microcomputer.

RESULTS

Interaction of FITC-Labeled Enzyme with Vanadate. The slow increase in fluorescence intensity (Pick, 1982; Pick & Karlish, 1982) following vanadate addition to FITC-labeled enzyme is characterized by pseudo-first-order kinetics (Figure 1). At a given pH, the pseudo-first-order rate constants k depend linearly on the vanadate concentration (Figure 2). The good fit of experimental results to a single exponential decay (Figure 1) and the linear dependence of k on [vanadate] (Figure 2) are consistent with the assumption that vanadate binding to FITC-labeled enzyme is the rate-determining step in the observed process and that the free enzyme and vanadate–enzyme complex each behave as a single enzyme species on the time scale of the experiment. The slope and intercept of the linear regression line (Figure 2) should represent the apparent rate constants for vanadate association to (k_{+1}) and dissociation from (k_{-1}) FITC-labeled enzyme. The ratio of the rate constants (k_{-1} = 0.21 min^{-1} and $k_{+1} = 3.1 \times 10^5$ M^{-1} min^{-1}) implies an equilibrium dissociation constant K_v = 0.66 μM, which is in good agreement with that calculated from the dependence of the extrapolated amplitude of the fluorescence change ($F_\infty - F_0$) on vanadate (Figure 2, inset).

The same dissociation constant could be measured independently under equilibrium conditions (15 min after vanadate addition). We have determined the dissociation equilibrium constant K_v at several pHs, by measuring the equilibrium

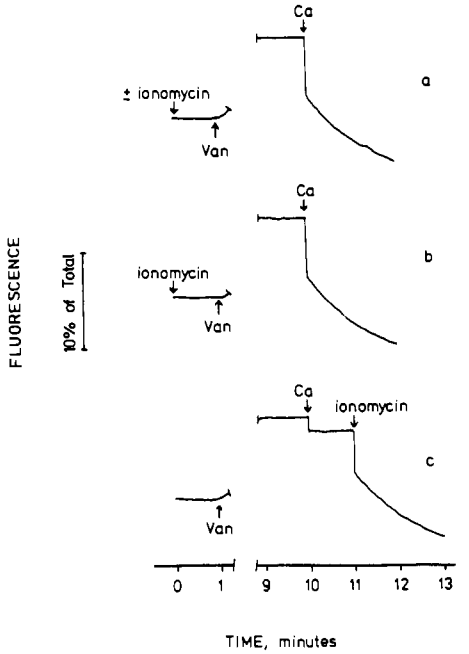


FIGURE 4: Comparison of the fluorescence response to Ca^{2+} of permeable and impermeable FITC-labeled SR vesicles. SR vesicles were labeled with FITC either under the standard conditions (pH 9.0 in the presence of EGTA) which lead to Ca^{2+}-permeable vesicles (a) or at low pH in the absence of EGTA (b and c). Vanadate (10 μM) was added to the vesicles in 2.5 mL of a medium as in Figure 1, in the absence or presence of 2 μg/mL ionomycin. After 10 min, 8 μL of 100 mM $CaCl_2$ was added (free $[Ca^{2+}]$ = 40 μM).

fluorescence amplitudes. The dependence of K_v on pH is summarized in Figure 3, with an example of the data at one pH shown in the insert. Protonation increases the affinity of the enzyme for vanadate. The line through the data of Figure 3 is the best fit to eq 1, where K_a represents the apparent

$$(K_v)_{app} = (K_v)_0(1 + K_a/[H^+]) \quad (1)$$

equilibrium constant for dissociation of a proton from the free enzyme or ligand. The apparent pK_a is 7–7.5. It should be noted that orthovanadate has a relevant dissociation (eq 2).

$$H_2VO_4^- \rightleftharpoons HVO_4^{2-} + H^+ \quad (2)$$

The pK for this vanadate ionization at zero ionic strength is 8.5 (Baes & Mesmer, 1976). Since the ionic strength of the solutions we have used is 0.12–0.30 M, the apparent pK for vanadate would be expected to be lowered; from Debye–Huckel theory it should be about 7.5–8.

Interaction of Ca^{2+} with FITC-Labeled Enzyme Preincubated with Vanadate. Rapid quenching of the fluorescence intensity is observed upon addition of calcium to FITC-labeled enzyme (Pick, 1982; Pick & Karlish, 1982; Highsmith et al., 1985), independent of the conditions used to label the vesicles (not shown). However, when calcium is added to vesicles preincubated with vanadate, the fluorescence response depends on the permeability of the vesicles to Ca^{2+} (Figure 4). With vesicles labeled at pH 7.0, in the absence of EGTA, a very small fluorescence quenching is observed (Figure 4c). Upon addition of the Ca^{2+}-ionophore ionomycin, biphasic quenching results. If ionomycin is added at the beginning of the experiment (Figure 4b), this biphasic quenching is seen directly upon addition of Ca^{2+}. On the other hand, vesicles labeled with FITC at pH 9.0 in the presence of EGTA (see Methods) show the strong biphasic response with or without ionomycin (Figure 4a). This demonstrates that the latter vesicles, which were used throughout the following experiments, are permeable

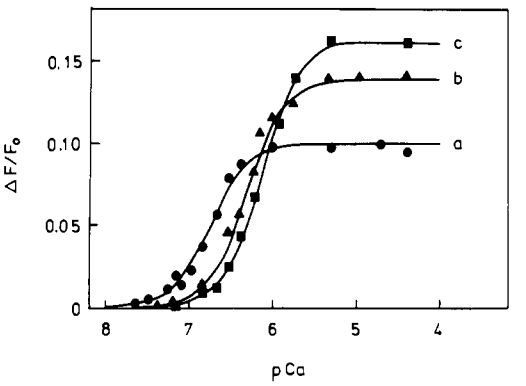


FIGURE 5: Dependence of the fluorescence amplitude change on Ca^{2+}. SR vesicles were preincubated 15 min in a medium as in Figure 1, with or without vanadate. The fluorescence intensity (516 nm) was measured before addition of Ca^{2+} and after equilibrium was reached (20 min after addition). Calcium concentrations given are final free $[Ca^{2+}]$. Lines are fits to eq 3. (a) No vanadate, $(K_{Ca})_{app}$ = 0.18 μM, N = 1.9; (b) 0.2 μM vanadate, $(K_{Ca})_{app}$ = 0.47 μM, N = 2.2; (c) 2 μM vanadate, $(K_{Ca})_{app}$ = 0.74 μM, N = 2.0.

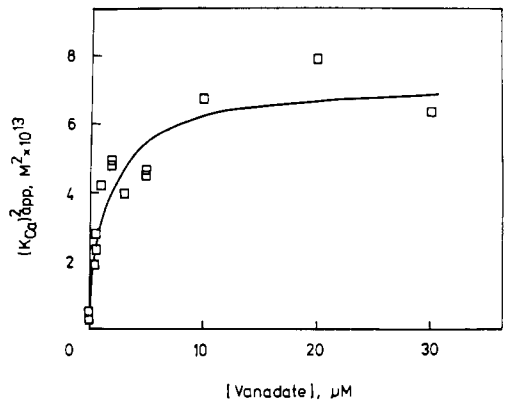
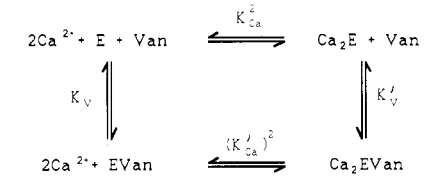


FIGURE 6: Dependence of $(K_{Ca})_{app}^2$ on vanadate. Replot of $(K_{Ca})_{app}^2$ derived as in Figure 5, as a function of [vanadate]. At saturating [vanadate], $(K_{Ca})_{app}^2$ approaches the asymptotic value 7×10^{-13} M^2.

Scheme I



to Ca^{2+} and that the response to Ca^{2+} of vesicles preincubated with vanadate depends on Ca^{2+} accessibility to the interior of the vesicles.

The amplitudes of both the fast and slow phases of the Ca^{2+}-induced fluorescence quenching increase with the Ca^{2+} concentration. At any constant vanadate concentration, the total amplitude change (ΔF, fast and slow phases) is cooperatively dependent on $[Ca^{2+}]$ (Figure 5) and can be fit very satisfactorily by eq 3. At the vanadate concentrations ex-

$$\Delta F = \frac{\Delta F_{max}[Ca^{2+}]^N}{(K_{Ca})_{app}^N + [Ca^{2+}]^N} \quad (3)$$

amined (0–40 μM) the Hill coefficient N is close to 2, which suggests that two (or more) calcium ions are involved in the observed change.

The apparent affinity of the enzyme for calcium is dependent on the vanadate concentration. It is convenient to express the affinity in terms of $(K_{Ca})_{app}^2$; the dependence of this derived quantity on [vanadate] is summarized in Figure

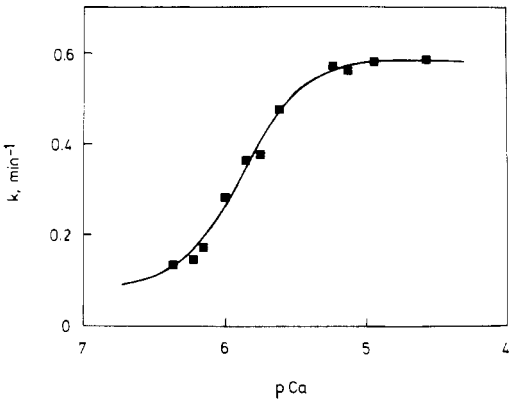


FIGURE 7: Dependence of the rate of calcium-induced fluorescence quenching on the calcium concentration. Vesicles were preincubated with 24 μM vanadate in a medium as in Figure 1, calcium was added to obtain the given final concentration, and the pseudo-first-order rate k for the slow phase of fluorescence decay (see Figure 4a) was determined. The curve is the best fit of the data to $k = k_{-2} + k_{+2}([Ca^{2+}]/K^*)^N/[1 + ([Ca^{2+}]/K^*)^N]$, where $k_{-2} = 0.084 \pm 0.045$ min^{-1}, $k_{+2} = 0.49 \pm 0.09$ min^{-1}, $K^* = (1.36 \pm 0.21) \times 10^{-6}$ M, and $N = 2.1 \pm 0.6$.

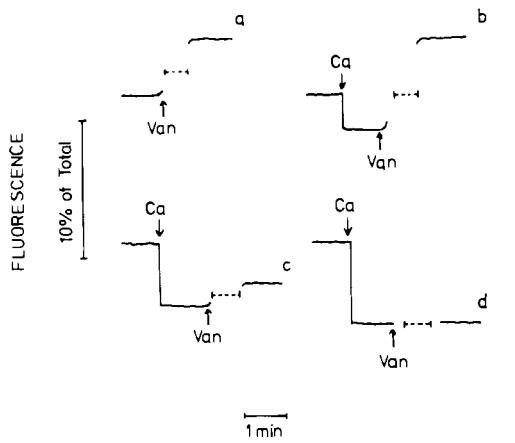


FIGURE 8: Fluorescence intensity changes upon addition of vanadate to FITC-labeled vesicles preincubated with calcium. FITC-labeled SR vesicles were preincubated as in Figure 1, with or without calcium. At the points indicated, 10 μM vanadate was added. The relative fluorescence intensity is shown before vanadate addition and after 15 min had passed (---). Free calcium concentrations were ~0 (a), 0.2 (b), 1 (c), and 11 μM (d).

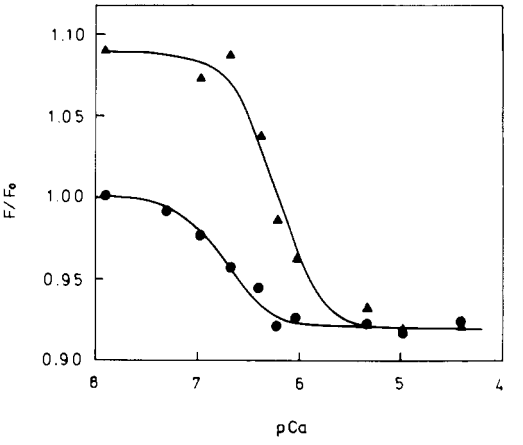


FIGURE 9: Fluorescence intensity of FITC-labeled SR vesicles at equilibrium, as a function of calcium concentration, in the absence and presence of vanadate. Experiments were carried out as in Figure 8, with varying calcium concentrations. The fluorescence intensity relative to that of free enzyme was measured at equilibrium after calcium addition (●) and after addition of 10 μM vanadate to vesicles preincubated with calcium (▲). The lines are cooperative binding isotherms, with $N = 2$, for $K_{Ca} = 0.19$ μM (lower curve) and $(K_{Ca})_{app} = 0.6$ μM (upper curve).

6. $(K_{Ca})_{app}^2$ reaches a constant level at high [vanadate], which requires that the simultaneous binding of Ca^{2+} and vanadate be possible (Scheme I). The value of $(K_{Ca})_{app}^2$ at saturating vanadate (7×10^{-13} M^2 at pH 7.0) should approach the constant for calcium dissociation from the calcium–enzyme–vanadate complex, $(K'_{Ca})^2$ (Scheme I). According to Scheme I, the ratio $(K'_{Ca}/K_{Ca})^2$ (where K_{Ca} is defined in the absence of vanadate; $K_{Ca}^2 = 5 \times 10^{-14}$ M^2) should be equal to K'_v/K_v. K_v was determined to be 0.6 μM in the experiments with FITC-labeled Ca^{2+}-ATPase described above, or 0.4 μM by inhibition of the initial velocity of the unlabeled ATPase (Markus et al., 1986), and K'_v was estimated to be 7 μM in the same ATPase experiments (Markus et al., 1986). It is satisfying that the ratios in question are very close, with both approximately 14.

Kinetics of Calcium Binding to the Enzyme–Vanadate Complex. The time dependence of the slow phase in the fluorescence quenching upon addition of calcium to the enzyme–vanadate complex can be fit well by a single exponential decay. At sufficiently high vanadate concentrations, the apparent rate constant (k) is independent of [vanadate]. The rate at high vanadate concentration shows saturating cooperative dependence on the concentration of calcium added (Figure 7). These kinetic results imply that the initial binding of calcium is to the enzyme–vanadate complex and that this is followed by a rate-determining unimolecular step (eq 4).

$$EVan + 2Ca^{2+} \underset{\text{fast}}{\overset{(K^*_{Ca})^2}{\rightleftharpoons}} Ca_2E^*Van \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} Ca_2EVan \quad (4)$$
slow

Several alternative kinetic mechanisms can be ruled out: Calcium binding to free enzyme in equilibrium with EVan would show an inverse dependence on [vanadate] at all vanadate concentrations. Rate-determining and effectively irreversible dissociation of vanadate before calcium binding would lead to an overall rate independent of calcium. The initial calcium binding cannot be the sole rate-determining step, since this would not lead to saturating dependence of the rate on $[Ca^{2+}]$. The Hill coefficient ($N = 2.1 \pm 0.6$) for the Ca^{2+} dependence suggests strongly that the species undergoing the slow transition has more than one Ca^{2+} bound.

Fluorescence Properties of the Ca_2EVan Complex. At high calcium concentrations (>10 μM free Ca^{2+}) the final equilibrium fluorescence level is nearly unaffected by vanadate, whether calcium is added to free enzyme or to enzyme preincubated with vanadate or vanadate is added to enzyme preincubated with calcium. The addition of vanadate to enzyme preincubated with lower calcium concentrations causes a slow enhancement of the fluorescence intensity, with the amplitude of the change increasing as $[Ca^{2+}]$ decreases (Figure 8).

The amplitude of the overall fluorescence change depends on $[Ca^{2+}]$, with $K_{Ca} \sim 0.19$ μM in the absence of vanadate and $K'_{Ca} \sim 0.6$ μM in the presence of saturating vanadate (10 μM, Figure 9). The agreement between K'_{Ca} determined here, where vanadate was added last, and that determined in the experiments shown in Figure 5, where Ca^{2+} was added last, indicates that the same equilibrium is reached in both cases.

Discussion

The results described here confirm the existence of a ternary complex of the SR Ca^{2+}-ATPase with vanadate and calcium, previously proposed on the basis of studies of the initial ATPase activity (Markus et al., 1986). They also provide evidence on

the pathway for formation of the complex and information on its relationship to other forms of the enzyme.

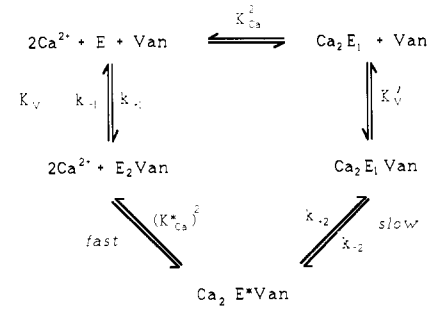
The fluorescence signal observed from an FITC-labeled SR preparation is an indicator of local conformational changes in the Ca^{2+}-ATPase (Pick, 1982; Pick & Karlish, 1982; Highsmith et al., 1985) and provides a convenient measure of the interaction of ligands with the enzyme. The vanadate-induced increase in fluorescence is as expected for a simple ligand-binding process. The values of the vanadate dissociation constant at pH 7.0 derived from the vanadate dependence of the rate of fluorescence change ($k_{-1}/k_{+1} = K_v$ = 0.66 μM, Figure 2) and from the amplitude of the change (either extrapolated, as shown in Figure 2 inset, or independently measured at equilibrium, K_v = 0.64 ± 0.15 μM) are in excellent agreement. The dissociation constant for the FITC-labeled enzyme is very similar to that previously calculated for the native enzyme from initial activity measurements (K_v = 0.41 ± 0.06 μM). It is important to note that the fluorescence response to vanadate shows no cooperativity (e.g., note Scatchard plot, Figure 3 inset). Although there is considerable evidence for a dimeric structure for the Ca^{2+}-ATPase in the native SR membrane (Scales & Inesi, 1976; Ikemoto et al., 1981; Moller, 1982; Silva & Verjovski-Almeida, 1983; Ikemoto & Nelson, 1984; Hymel et al., 1984), there is no clear agreement as to whether there is any functional significance to the dimeric structure (Jorgensen et al., 1978; Dean & Tanford, 1978; Moller et al., 1980; Ikemoto & Nelson, 1984; Froehlich & Heller, 1985; Lund & Moller, 1988). The results presented here imply that the conformational changes induced by vanadate binding to each monomer are completely independent.

One important question is the identity of the bound vanadate species, since vanadate is known to form oligomeric metavanadates and decavanadate (Baes & Mesmer, 1976). From equilibrium constants in the literature (Baes & Mesmer, 1976), however, it can be shown that a stock solution of 10^{-3} M sodium orthovanadate at pH 10 exists nearly exclusively in the form HVO_4^{2-}, with the most abundant oligomeric species $V_3O_9^{3-}$ present in less than one part in 10^4. On dilution into an SR solution at the typical vanadate concentrations used, $[V_3O_9^{3-}]$ would be less than 10^{-9} M, much lower than the enzyme site concentration ($\sim 2 \times 10^{-8}$ M). The polymerization–depolymerization reactions of vanadate species are slow ($t_{1/2}$ of several hours at room temperature) (Kustin & Macara, 1982), so it is unlikely that the processes observed (e.g., Figure 2) could involve polymerization of vanadate driven by binding of an oligomer species to an enzyme site. Finally, the calculated concentrations of the oligomeric species are so low that their association rate constants would have to be far above the diffusion control limit for them to be responsible for any of the processes reported here.

The pH dependence of vanadate binding in the absence of calcium (Figure 3) shows that dissociation of a proton with an apparent pK near 7–7.5 from the free enzyme or from free HVO_4^{2-} weakens the association between them. Since HVO_4^{2-} ionizes in this pH range, it is likely that HVO_4^{2-} itself is the strongly bound species. Detailed studies of the pH dependence of enzyme phosphorylation from P_i have shown that at least two protonations are involved in stabilizing the phosphoenzyme (Inesi et al., 1984).

It has been observed previously (Pick, 1982; Pick & Karlish, 1982) that, in the presence of saturating Ca^{2+} concentrations, vanadate has very little effect on the final intensity of the fluorescence of FITC-labeled SR. These observations have been interpreted as demonstrating that Ca^{2+} and vanadate binding to the SR Ca^{2+}-ATPase are exclusive. However, we have shown that a calcium–enzyme–vanadate complex exists (Scheme I). K'_v is about 7 μM, so that at saturating Ca^{2+} concentration and, e.g., 10 μM vanadate (see Figures 8 and 9) the ternary complex is the major enzyme species at equilibrium. The only other significant species under these conditions is Ca_2E. The similarity of the fluorescence in the absence and presence of vanadate thus implies that the environment of the fluorophore in Ca_2E and Ca_2EVan is nearly the same. The conformation of the nucleoside binding domain [the site of bound fluorescein (Pick & Karlish, 1980; Pick & Bassilian, 1981; Mitchison et al., 1982)] in the ternary complex must then be similar to that of Ca_2E and different from that of EVan—that is, "E_1-like" rather than "E_2-like" (deMeis & Vianna, 1979; Jencks, 1980).

Scheme II



The kinetics of the fluorescence changes observed on addition of Ca^{2+} to enzyme preincubated with vanadate are consistent with the reaction pathway of eq 4, that is, a slow conformational change ($t_{1/2}$ > 1 min) follows rapid Ca^{2+} binding ($t_{1/2}$ < 2 s) to the EVan complex. Since these changes are only observed when the vesicles are permeable to Ca^{2+} (Figure 4), the sites to which Ca^{2+} binds must only be accessible from the interior of the vesicle. This is as expected if vanadate stabilizes the enzyme in the "E_2 conformation", which has (low-affinity) Ca^{2+} binding sites accessible only to the SR lumen, e.g., if EVan is analogous to "E_2P" (O'Neal et al., 1979; Inesi et al., 1980; Dupont & Bennet, 1982; Dux & Martonosi, 1983; Medda & Hasselbach, 1983; Inesi et al., 1984; Andersen & Moller, 1985; Medda & Hasselbach, 1986). The overall mechanism for Ca^{2+} and vanadate binding can thus be summarized in Scheme II, in which Ca_2E^*Van arises from rapid, cooperative (N = 2) calcium binding to E_2Van and slowly rearranges to the more stable Ca_2E_1Van.

From Scheme II, the apparent first-order rate constant for the slow phase of the fluorescence decay observed, when Ca^{2+} is added to enzyme preincubated with a saturating concentration of vanadate, should be given by eq 5.

$$k = k_{-2} + k_{+2} \frac{([Ca^{2+}]/K^*)^2}{1 + ([Ca^{2+}]/K^*)^2} \quad (5)$$

The fit of the observed data (e.g., Figure 7) to this equation leads to estimates of 0.49 and 0.08 min^{-1} for k_{+2} and k_{-2}, respectively, and $K^*_{Ca} = 1.4 \times 10^{-6}$ M. The conformational equilibrium favors Ca_2E_1Van by k_{+2}/k_{-2} = 6-fold. [Note that within experimental error $(K^*_{Ca})^2 \times k_{-2}/k_{+2} = (K'_{Ca})^2$, as expected.]

The two slowly interconverting species Ca_2E^*Van and Ca_2E_1Van are thus apparently *isomeric* states of the SR Ca^{2+}-ATPase which may be E_2-like and E_1-like, respectively. The phosphoryl enzyme species to which Ca_2E^*Van may be analogous, Ca_2E_2–P, is postulated as the species in the transport cycle from which Ca^{2+} is dissociated to the SR lumen

(Takisawa & Tonomura, 1979; deMeis & Vianna, 1979). Ca_2E_2–P apparently does not accumulate under any conditions (Jencks, 1980; Inesi & Watanabe, 1982; Takisawa & Makinose, 1983). In fact, although the assumption of multiple forms of Ca–E–P has been found useful for explaining the complexities of various kinetic observations [e.g., Fernandez-Belda et al. (1984), Froehlich and Heller (1985), and Nakamura et al. (1986)], there is little direct evidence for the existence of the two discrete states Ca_2E_1–P and Ca_2E_2–P (Fernandez-Belda & Inesi, 1986; Stahl & Jencks, 1987; Khananshvili & Jencks, 1988). The slow interconversion of the calcium–enzyme–vanadate species, on the other hand, makes it possible to directly observe Ca_2E^*Van. It is not clear why the conversion of Ca_2E^*Van to Ca_2E_1Van should be slow; one possibility is that it involves the making and/or breaking of covalent bonds, for which the enzyme is unable (in this case) to provide acceleration.

It is of interest to note that there is relatively little mutual destabilization of bound vanadate by bound calcium. The ratio of the dissociation constants for one species in the presence and absence of the other [K'_v/K_v or $(K'_{Ca}/K_{Ca})^2$] is only about 14; the interaction energy between the bound ligands is thus only about 1.6 kcal/mol. Pickart and Jencks (1984) have calculated that phosphate and calcium bound to the Ca^{2+}-ATPase mutually destabilize one another by 7.6 kcal/mol. This mutual destabilization is critical for rapid ion transport by the SR Ca^{2+}-ATPase, since it allows dissociation of Ca^{2+} inside the SR even as the internal Ca^{2+} concentration builds up. The weak mutual destabilization between vanadate and calcium means, of course, that the internal calcium sites of Ca_2E^*Van have a relatively high affinity for Ca^{2+} (K^*_{Ca} = 1.4 μM). Since it is not known how phosphate binding destabilizes bound calcium (Pickart & Jencks, 1984), little can be said about the difference between phosphate and vanadate in this regard. What is clear is that the calcium sites are not necessarily weakened simply by being accessible to the SR lumen as opposed to the cytosolic side of the membrane but rather by a quite specific effect of covalently bound phosphate, which is poorly mimicked by vanadate.

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Registry No. ATPase, 9000-83-3; Van, 14333-18-7; Ca, 7440-70-2.

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Characterization of the Ethenoadenosine Diphosphate Binding Site of Myosin Subfragment 1. Energetics of the Equilibrium between Two States of Nucleotide·S1 and Vanadate-Induced Global Conformation Changes Detected by Energy Transfer†

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ABSTRACT: The fluorescence decay of 1,N^6-ethenoadenosine diphosphate (ϵADP) bound to myosin subfragment 1 (S1) was studied as a function of temperature. The decay was biexponential, and the two lifetimes were quenched relative to the single lifetime of free ϵADP. The temperature dependence of the fractional intensities of the decay components showed two states of the S1·ϵADP complex. At pH 7.5 in 30 mM TES, 60 mM KCl, and 3 mM $MgCl_2$, the equilibrium constant for the conversion of the low-temperature state ($S1_L$·ϵADP) to the high-temperature state ($S1_H$·ϵADP) was 40 at physiological temperatures, and $\Delta H°$ = 13 kcal·mol^{-1} and $\Delta S°$ = 49 cal·deg^{-1}·mol^{-1}. At 10 °C the equilibrium constant of S1 for ϵADP was 5, indicating that $S1_H$·ϵADP was the dominant state, and that for the vanadate complex ϵADP·Vi was 0.7, suggesting that in S1·ϵADP·Vi the dominant state of the S1–nucleotide complex was converted from $S1_H$·ϵADP to $S1_L$·ϵADP. The single rotational correlation time of bound ϵADP at 10 °C decreased from 107 ns in S1·ϵADP to 74 ns in $S1^+$·ϵADP·Vi. Conversion of the binary complex to the ternary vanadate complex resulted in a 3-Å decrease in the energy transfer distance between bound ϵADP and *N*-[4-(dimethylamino)-3,5-dinitrophenyl]maleimide attached to SH_1 and a decrease of the average distance between bound ϵADP and bound Co^{2+} from 12.6 to 8.3 Å. On the assumption that $S1^+$·ADP·Vi is a good stable analogue of S1·ADP·P_i, it is suggested that the transition $S1_L$·ϵADP → $S1_H$·ϵADP is involved in the power stroke of the contractile cycle. The structural changes that S1 experiences during this transition may include a small increase in dimensional asymmetry and movements of two regions of the heavy chain toward the adenine-binding site.

Elucidation of the mechanism of the cyclic interactions of myosin with ATP and actin requires detailed knowledge of the conformation of myosin subfragment 1 at which the nucleotide- and actin-binding sites are located. The knowledge must include the gross conformation of S1[1] and the conformations of the ATPase site and the actin-binding sites. Several fluorescent nucleotide analogues are available for investigation of nucleotide binding to a variety of proteins. The 1,N^6-ethenoadenosine derivatives are particularly well suited for this purpose because of a high degree of structural similarity to the parent adenine-containing compounds and favorable spectral properties that minimize background interference arising from aromatic amino acid residues. We (Garland & Cheung, 1976, 1979) previously investigated the kinetics and mechanism of the binding of ϵADP and ϵATP to S1 and heavy meromyosin and showed that the binding constants of S1 for ϵADP obtained from equilibrium dialysis and kinetic experiments were in good agreement with each other and with those obtained for ADP by other investigators. The early association constants of S1 for ϵADP were recently confirmed in an equilibrium polarization study by Perkins et al. (1984a).

The orthovanadate ion is an inhibitor of myosin ATPase. Together with ADP, Vi binds stoichiometrically to the active site of the enzyme and forms an inactive complex (Goodno, 1982). On the basis of the kinetics of ATPase inhibition, it

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[1] Abbreviations: S1, myosin subfragment 1; ϵADP, 1,N^6-ethenoadenosine diphosphate; ϵATP, 1,N^6-ethenoadenosine 5′-triphosphate; Vi, orthovanadate ion; DDPM, *N*-[4-(dimethylamino)-3,5-dinitrophenyl]-maleimide; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; FRET, fluorescence resonance energy transfer; IAEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine.

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